

## Internal Cleavage of Hepatitis C Virus NS3 Protein Is Dependent on the Activity of NS34A Protease

Se-Hwan Yang,\* Chang Geun Lee,\* Man Ki Song,\* and Young Chul Sung\*<sup>†1</sup>

\*Department of Life Science and <sup>†</sup>School of Environmental Engineering, Pohang University of Science and Technology, Pohang, Kyungbuk, Republic of Korea

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The nonstructural protein NS3 of the hepatitis C virus (HCV) is indispensable for virus replication and a multifunctional enzyme that contains three catalytic activities such as serine protease, helicase, and NTPase. Here, we demonstrated that the internal cleavage of the HCV NS3 protein occurs in various mammalian cells such as HepG2, COS-7, and NIH3T3. As is observed for the internal cleavage mechanism of the NS3 protein of dengue virus 2, the internal processing of HCV NS3 protein was catalyzed by the active NS3 serine protease and NS4A, but not NS3 alone. From the data acquired from extensive site-directed mutagenesis, we observed that the NS3 protein was internally cleaved at two different sites, FCH<sup>1395</sup>[S<sup>1396</sup>KK and IPT<sup>1428</sup>[S<sup>1429</sup>GD, within RNA helicase domain. The internal cleavage of NS3 protein by NS34A protease was also confirmed in a different isolate of HCV-1b strain. In addition, *in vitro* transforming assays demonstrated that the internal cleavage product of NS3, NS3a-1, appeared to have higher oncogenic potential than does intact NS3. Taken together, our results suggest that the internal cleavage of NS3 may be associated with the replication and oncogenesis of HCV. © 2000

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### INTRODUCTION

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and accounts for the majority of posttransfusion and sporadic non-A, non-B viral hepatitis worldwide (Choo *et al.*, 1989). The persistence of HCV in infected humans leads to cirrhosis and hepatocellular carcinoma (Kiyosawa *et al.*, 1990). The viral genome is a single-stranded, positive-sense RNA of approximately 9.5 kb, which encodes a polyprotein precursor. This polyprotein is cleaved by host-signal peptidase and two virally encoded proteases to generate viral structural (core, E1, and E2/p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), respectively (Hijikata *et al.*, 1991).

The viral serine protease resides in the amino-terminal half of the NS3 protein, which, with NS4A as a cofactor, cleaves NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (Bartenschlager *et al.*, 1993; Tomei *et al.*, 1993). In addition to enzymatic activity, it has been reported that the 5' region of HCV NS3 transforms NIH3T3 cells (Sakamuro *et al.*, 1995) and suppresses actinomycin-D-induced apoptosis of NIH3T3 cells (Fujita *et al.*, 1996), suggesting that the NS3 may play important roles on

pathogenic effects in host cells infected by HCV. Furthermore, the carboxyl-terminal half of the NS3 protein has a nucleotide triphosphate-RNA helicase activity (Kim *et al.*, 1995). Therefore, NS3 is a multifunctional protein that contributes to virus replication.

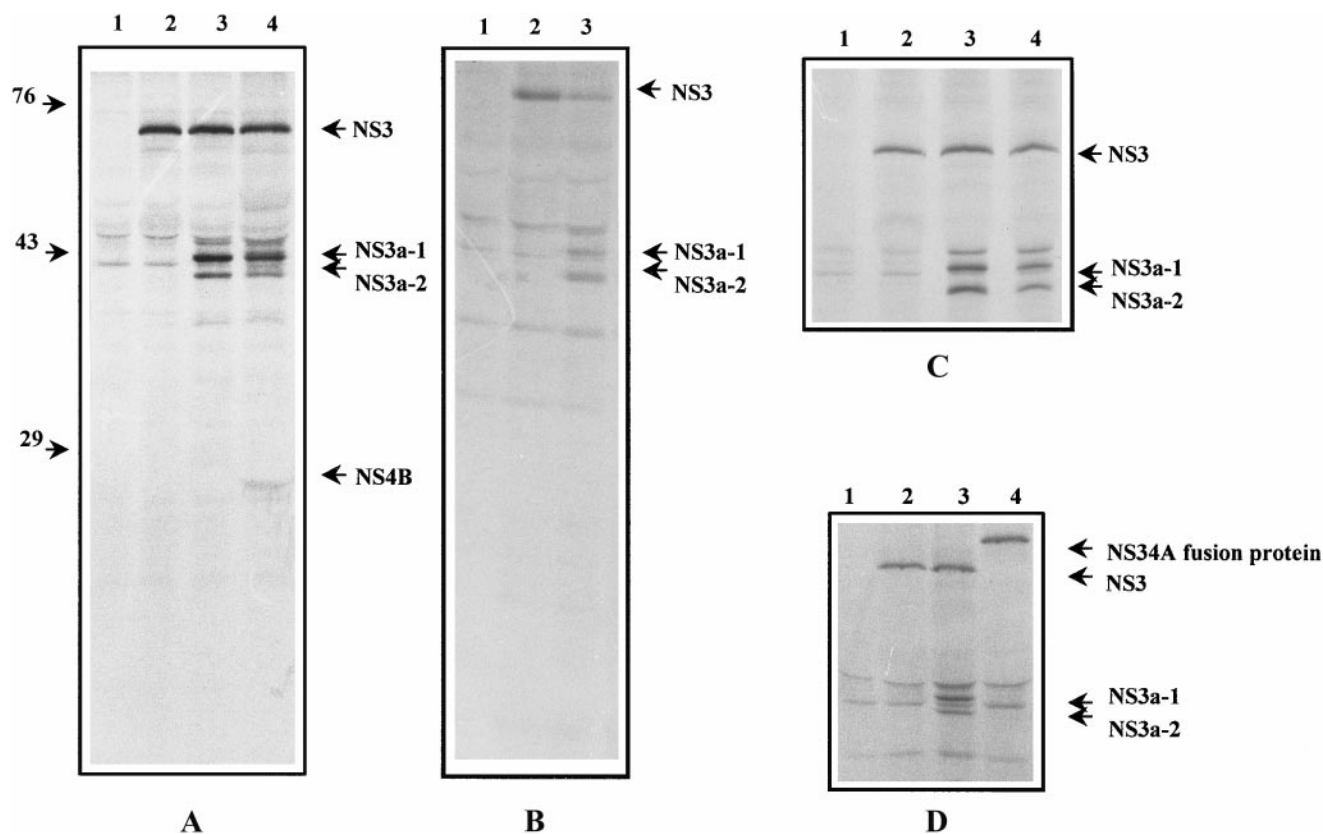
It was recently reported that the NS3 protein is internally cleaved within an RNA helicase sequence motif in insect and 293T cells not by NS3 protease, but by a cellular protease (Shoji *et al.*, 1999). In contrast, we found that the internal processing of the NS3 protein is dependent on the NS3 protease activity in a variety of mammalian cell types. Moreover, internal cleavage of NS3 was shown to require the presence of NS4A cofactor. In addition, we demonstrated that the internal cleavage product of NS3 has higher oncogenic potential than does the intact NS3 protein and further increases the oncogenic activity of the HCV core. These results suggest that the internal cleavage product of NS3 may play a role in the replication and oncogenesis of HCV.

### RESULTS AND DISCUSSION

#### Internal processing of HCV NS3 by the activity of NS34A serine protease

To express the NS3 (amino acids [aa] 1019–1657), NS34A (aa 1019–1711), and NS3-NS4-NS5 (aa 1019–3010) coding region, COS-7 cells were transfected with pNS3, pNS34A, and pNS345 plasmids, respectively, which were derived from HCV-1b (JS strain). By immunoprecipitation using anti-NS3 and anti-NS4 positive patients' sera, the

<sup>1</sup> To whom correspondence and reprint requests should be addressed at Pohang University of Science and Technology, Department of Life Science, San 31 Hyoja-Dong, Nam-Gu, Pohang, Kyungbuk 790-784, Republic of Korea. Fax: 82-562-279-5544. E-mail: ycsung@postech.ac.kr.



**FIG. 1.** The internal cleavage of HCV NS3 protein requires both NS4A and NS3 serine protease activity. (A) COS-7 cells transfected with pCI-neo (lane 1), pNS3 (lane 2), pNS34A (lane 3), or pNS345 (lane 4) were labeled with [ $^{35}$ S]methionine and immunoprecipitated with pooled HCV-infected human sera and electrophoresed through an SDS-12% polyacrylamide gel. The molecular sizes (kDa) are indicated on the left. Arrows on the right indicate the full-size NS3 protein (72 kDa) (lanes 3 and 4), its cleavage products, NS3a-1 (44 kDa) and NS3a-2 (41 kDa) (lanes 3 and 4), and NS4B protein (26 kDa) (lane 4). (B) pCI-neo- (lane 1), pNS3- (lane 2), or pNS34A (lane 3)-transfected COS-7 cells were immunoprecipitated with antiprotease Ab. (C) NIH3T3 transfected with pNS4A (lane 1), pNS3 (lane 2), pNS34A (lane 3), or pNS3 and pNS4A (lane 4) were analyzed by immunoprecipitation using antiprotease Ab. (D) HepG2 cells transfected with pCI-neo (lane 1), pNS3 (lane 2), pNS34A (lane 3), or pNS34A-M1 (lane 4) were immunoprecipitated with antiprotease Ab. The unprocessed-NS34A fusion protein (80 kDa), full-size NS3 protein (72 kDa), and its processed products, NS3a-1 (44 kDa) and NS3a-2 (41 kDa) are indicated by arrows.

expression and processing pattern of the NS3 were investigated. As a result, we found that the NS3 protein expressed from pNS34A and pNS345, but not pNS3, was processed to generate two different products, which were designated as NS3a-1 (44 kDa) and NS3a-2 (41 kDa) (Fig. 1A, lanes 2–4). NS3a-1 and NS3a-2 bands in lane 4 seemed fainter than those seen in lane 3. However, the NS3 band was also slightly fainter than that in lane 3, indicating that this different observation between lane 3 and lane 4 was the result of the difference of the expression level of NS3 protein derived from pNS34A- or pNS345-expression plasmids. Therefore, these results indicate that internal cleavage of NS3 occurs in NS345 polyprotein as well as NS34A itself and that NS4A is indispensable for internal processing of NS3. In pNS345 transfectants, NS5A and NS5B protein were not detected (lane 4), which is the result of low anti-NS5 antibody titer. A faint band reactive to the anti-NS3 positive sera at the position moving slightly slower than NS3a-1 could be detectable (lanes 3 and 4), implying that this band may

also correspond to one among internal cleavage products. However, we focused on the characterization of two different products, NS3a-1 and NS3a-2, because of its amount of very low level compared to that of NS3a-1 or NS3a-2.

To investigate whether NS3a-1 and NS3a-2 corresponded either to the N-terminal or the C-terminal portion of NS3, COS-7 cells transfected with pNS3 and pNS34A were immunoprecipitated with antibody recognizing the serine protease domain of NS3 (antiprotease antibody). The polyclonal antiprotease antibody was raised against the recombinant serine protease domain of NS3 (aa 1027–1218), expressed, and purified in *Escherichia coli*. Both NS3a-1 and NS3a-2 proteins were detected with antiprotease antibody (Fig. 1B, lane 3), indicating that these cleavage products correspond to the N-terminal portion of the NS3 protein.

To determine whether the internal cleavage of NS3 also occurs when NS4A proteins were provided in *trans*, NIH3T3 cells were cotransfected with both pNS3 and

pNS4A expression plasmid. The transfected cells were analyzed by immunoprecipitation with antiprotease antibody. When NS4A were provided in *trans* (Fig. 1C, lane 4), the internal cleavage of NS3 occurred at a slightly lower efficiency than that derived from pNS34A (Fig. 1C, lane 3). It has been reported that the structure of the catalytic site of the NS34A protease is significantly different from that of the NS3 protease (NS3<sup>pro</sup>), indicating that the binding of NS4A to NS3 induces tertiary structure changes, resulting in accelerated enzyme reactions (Steinkuhler *et al.*, 1996; Kwong *et al.*, 1998). It is therefore possible that NS4A induces the conformational change of the catalytic site of NS3, which is required for the internal cleavage by NS3<sup>pro</sup>.

We next tested whether the serine protease activity of HCV NS3 is necessary for the internal processing of the NS3 protein. It was reported that the activity of NS3 protease requires a catalytic triad: His<sup>1083</sup>, Asp<sup>1107</sup>, and Ser<sup>1165</sup> (Bazan *et al.*, 1989). To inactivate NS3 serine protease, Ser<sup>1164</sup> and Ser<sup>1165</sup> in the NS3 region were mutated to Ala and Ala by site-directed mutagenesis and the resulting plasmid, pNS34A-M1, was transfected to human hepatoma HepG2 cells. The unprocessed-NS34A fusion protein (80 kDa), but not NS3a-1 and NS3a-2, was detected by immunoprecipitation with antiprotease antibodies (Fig. 1D, lane 4), indicating that serine protease activity is required for internal cleavage of NS3. The observation that NS3 and NS34A-M1 protein were not internally cleaved by themselves indicates that there is no cellular protease capable of internal cleavage of NS3. These results indicate that the internal cleavage of HCV NS3 protein requires not only NS4A as a cofactor but also the activity of NS3 serine protease. However, there is still another possibility that NS34A protease may activate a cellular protease and the activated protease is responsible for the internal cleavage of NS3.

Our observations are similar to the internal cleavage of NS3 derived from dengue virus 2 (DEN-2), a member of the *Falvivirus* family. The internal cleavage of NS3 in DEN-2 is dependent on the serine protease activity in the NS3 region as well as NS2B as a cofactor (Falgout *et al.*, 1991; Arias *et al.*, 1993; Keng *et al.*, 1997).

In contrast to these results, it was recently reported that the internal processing of the NS3 protein occurred within the RNA helicase sequence motif (amino acid position, QRR<sup>1488</sup>|GRT) in insect cells and 293T cells, and is mediated by a cellular protease (Shoji *et al.*, 1999). These conflicting observations may stem from the following reasons: (1) the internal processing of the NS3 by cellular protease occurs only in specific cell types, such as insect cells and 293T cells; and (2) the products of NS3 cleaved by cellular protease may be unstable in COS-7, NIH3T3, and HepG2 cells.

As shown in Fig. 1A, we could not detect the C-terminal counterparts (proteins of 28 and 31 kDa) of NS3a-1 (44 kDa) and NS3a-2 (41 kDa) by immunoprecipi-

tation. Pulse-chase analysis indicated that NS3a-1 and NS3a-2 were stable during a 120-min chase period, but that the C-terminal cleavage products of these proteins were not detectable (data not shown). It has been previously shown that the C-terminal cleavage product of NS3 derived from DEN-2 virus was not detected at the 60-min chase in transfected cells, although it was faintly detectable in a 10-min chase (Keng *et al.*, 1997). Thus, it is likely that the C-terminal cleavage products of HCV NS3 are too unstable to be detected by using standard procedures. Alternatively, it is also possible that the failure to detect the C-terminal moieties of the NS3 may result from the used antibodies with low reactivity to the short C-terminal piece of the NS3 protein.

### Mutational analysis of the putative cleavage sites of NS3

Immunoprecipitation data using antiprotease antibody led us to infer that the internal cleavage of NS3 protein occurred at two different sites, and that the NS3a-1 and NS3a-2 proteins belonged to the N-terminal portion of NS3. In DEN-2 virus, the processing events by the NS3 protease (NS3<sup>pro</sup>) occurred in regions containing a dibasic amino acid motif (Lys or Arg) at the P1 and P2 positions, followed by small branched residues (Ser, Ala, or Gly) at the P1' position (Falgout *et al.*, 1991; Arias *et al.*, 1993). According to the nomenclature of Berger and Schechter (1970), P1 and P1' are designated as the carboxy- and amino-terminus generated by cleavage of the peptide bond, respectively. Since the internal cleavage of NS3 proteins by the NS2B/NS3 complex of DEN-2 was demonstrated to occur at the site RR<sup>458</sup>|G<sup>459</sup>R, which corresponds to the conserved cleavage site of DEN-2 NS3<sup>pro</sup>, it is likely that the internal cleavage of HCV NS3 protein also occurs in regions that show sequence homology with the conserved cleavage sites of HCV NS3<sup>pro</sup>. It was known that conserved cleavage sites of NS3<sup>pro</sup> of HCV consist of an acidic residue at P6, threonine or cysteine at P1, and serine or alanine at P1' (Komoda *et al.*, 1994; Steinkuhler *et al.*, 1996). On the basis of either the sequence homology of cleavage sites of NS3<sup>pro</sup> or the size of NS3a-1 and NS3a-2, we have constructed 14 NS34A point-mutants (from pNS34A-M2 to pNS34A-M15) (Fig. 2A). To generate a restriction enzyme site and completely block the internal cleavage, two amino acids at putative P1 and P1' residues were changed to either Ala, Cys, or to Pro, Gly (Fig. 2A). When NIH3T3 cells were transfected with these NS34A mutants, the internal cleavage of NS3 was blocked only in cells transfected with pNS34A-M5 and pNS34A-M13 (Fig. 2A). NS3a-2 was not detected in cells transfected with pNS34A-M5 (H<sup>1395</sup>|S<sup>1396</sup> → P|G), while full-size NS3 and NS3a-1 proteins were detected (Fig. 3A, lane 4). In addition, NS3a-1 was not detected in pNS34A-M13 (T<sup>1428</sup>|S<sup>1429</sup> → A|S) transfected cells (Fig. 3A, lane 5). These results indicate

**A**

Plasmid DNA transfected	Amino acid substitutions	Internal cleavage activity	
		H <sup>1395</sup> S <sup>1396</sup>	T <sup>1428</sup> S <sup>1429</sup>
pNS3		-	-
pNS3 + pNS4A		+	+
pNS34A		+	+
pNS34A-K		+	+
pNS34A-M1	1164, 1165; Ser, Ser → Ala, Ala	-	-
pNS34A-M2	1305, 1306; Cys, Ser → Ala, Cys	+	+
pNS34A-M3	1324, 1325; Thr, Ser → Ala, Cys	+	+
pNS34A-M4	1392, 1393; Ile, Phe → Pro, Gly	+	+
pNS34A-M5	1395, 1396; His, Ser → Pro, Gly	-	+
pNS34A-M6	1398, 1399; Lys, Lys → Pro, Gly	+	+
pNS34A-M7	1399, 1400; Lys, Ser → Ala, Cys	+	+
pNS34A-M8	1400, 1401; Asp, Glu → Pro, Gly	+	+
pNS34A-M9	1403, 1404; Ala, Ala → Pro, Gly	+	+
pNS34A-M10	1406, 1407; Leu, Ser → Pro, Gly	+	+
pNS34A-M11	1406, 1407; Leu, Ser → Ala, Cys	+	+
pNS34A-M12	1409, 1410; Leu, Gly → Pro, Gly	+	+
pNS34A-M13	1428, 1429; Thr, Ser → Ala, Cys	+	-
pNS34A-M14	1525, 1526; Cys, Ala → Ala, Cys	+	+
pNS34A-M15	1536, 1537; Thr, Ser → Ala, Cys	+	+

**B**

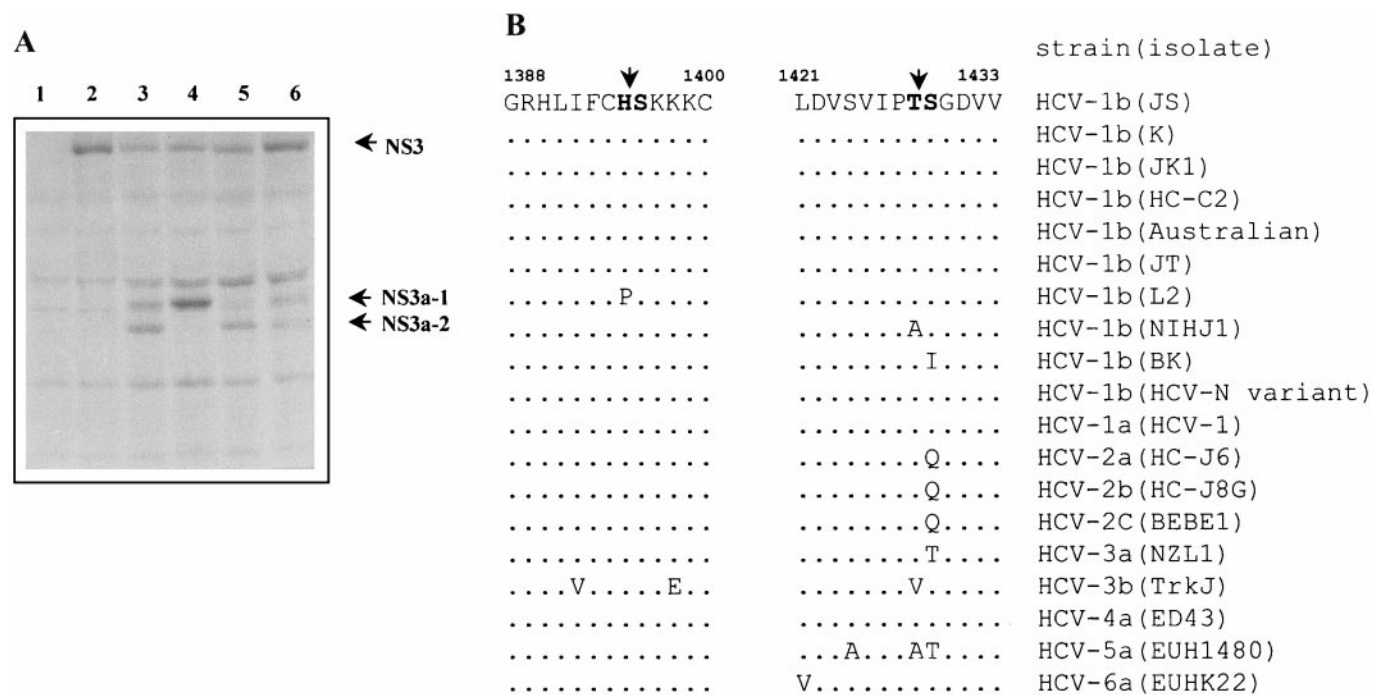
**FIG. 2.** (A) The names of NS3-, NS4A-, NS34A-, or mutant NS34A-encoding plasmids are shown on the left. Using site-directed mutagenesis, 15 NS34A mutants are generated to analyze the internal cleavage sites of the HCV NS3 protein. The positions of amino acid substitution and converted amino acids are indicated. The internal cleavage activity of each mutant at the two different sites, H<sup>1395</sup>S<sup>1396</sup> and T<sup>1428</sup>S<sup>1429</sup>, is indicated with + or -, where + = cleavage and - = no detectable cleavage. (B) Schematic representation of nonstructural region of HCV. Vertical arrows indicate the NS3 protease-mediated cleavage sites. Curved arrows indicate the internal cleavage sites mediated by the NS34A protease. The numbers on the bars indicate the positions of amino acids.

that the internal cleavage sites of NS3 by the NS34A protease are located at amino acid residues 1395/1396 and 1428/1429, which are located in the RNA helicase domain (Fig. 2B). When the internal cleavage site (DVS-VIPT<sup>1428</sup>/S<sup>1429</sup>) was compared with the conserved cleavage sites of NS3<sup>pro</sup>, amino acid residues of P1 and P1' positions (Thr and Ser) exactly coincided with those of the cleavage site of NS3<sup>pro</sup>. Acidic residue (Asp), however, is found at the P7 position rather than at the P6 position. Analysis of the other internal cleavage site (RHLIFCH<sup>1395</sup>/S<sup>1396</sup>) showed that amino acid residue (Ser) of P1' position is coincided, but amino acid residues at P1, P6 positions are different from those of the conserved cleavage sites of NS3<sup>pro</sup>. Since it was demonstrated that cleavage sites of NS3<sup>pro</sup> have high flexibility for substitution at P1, P1', and P6 positions (Kolykhalov *et al.*, 1994; Bartenschlager *et al.*, 1995), it is not surprising that the

internal cleavage sites of NS3 identified in this study are somewhat different from the conserved cleavage sites. Recently, Kang and Oh (1998) have determined the X-ray crystallographic structure of the helicase domain of the NS3 protein. According to these data, the internal cleavage sites of NS3 are exposed on the surface of an arginine-rich domain in the helicase region, suggesting that internal cleavages could occur as a favorable interaction with the active site of the NS3 protease (personal communication).

To investigate whether these internal cleavage sites were conserved in other genotypes of HCV, the potential internal cleavage sites in various HCV genotypes and isolates were analyzed. A sequence alignment indicated that the internal cleavage site, HLIFCH<sup>1395</sup>/S<sup>1396</sup> was highly conserved in most HCV genotypes, whereas the DVS-VIPT<sup>1428</sup>/S<sup>1429</sup> site was less conserved (Fig. 3B). To





**FIG. 3.** (A) Analysis of the putative cleavage sites in the NS3 protein. NIH3T3 cells transfected with pCI-neo (lane 1), pNS3 (lane 2), pNS34A (lane 3), pNS34A-M5 (lane 4), pNS34A-M13 (lane 5), or pNS34A-K (lane 6) were immunoprecipitated with an antiprotease antibody and electrophoresed through an SDS-12% polyarylamide gel. The full-size NS3 protein as well as NS3a-1 and NS3a-2 are indicated by arrows on the right. (B) Sequence alignment of the internal cleavage site in NS3 of various HCV genotypes. The numbers on the sequence indicate the position of the amino acids of HCV NS3. Arrows on the top indicate the putative internal cleavage sites in NS3. A dot indicates a residue that is identical to that in HCV 1b (JS). The HCV isolates used for alignments are as follows: JS (Sugiyama *et al.*, 1997), K (Cho *et al.*, 1993), JK1 (Honda *et al.*, 1992), HC-C2 (Wang *et al.*, 1993), Australian (Trowbridge *et al.*, 1998), JT (Tanaka *et al.*, 1992), L2 (Cho *et al.*, 1995), NIHJ1 (Aizaki *et al.*, 1998), BK (Takamizawa *et al.*, 1991), HCV-N variant (Hayashi *et al.*, 1993), HCV-1 (Choo *et al.*, 1991), HC-J6 (Okamoto *et al.*, 1991), HC-J8G (Okamoto *et al.*, 1992), BEBE1 (Nakao *et al.*, 1996), NZL1 (Sakamoto *et al.*, 1994), TrkJ (Chayama *et al.*, 1994), ED43 (Chamberlain *et al.*, 1997a), EUH1480 (Chamberlain *et al.*, 1997b), and EUHK22 (Adams *et al.*, 1997).

determine whether the internal processing of NS3 protein also occurred in other isolates of HCV, we constructed pNS34A-K derived from the PCR-amplified NS34A region of HCV-1b (K isolate) (Cho *et al.*, 1993), which has 92.5% amino acid sequence homology with pNS34A within the NS3 region and possesses putative internal cleavage sites (Fig. 3B). As expected, NS3a-1 and NS3a-2 were also detected in NIH3T3 cells transfected with pNS34A-K (Fig. 3A, lane 6), indicating that the internal processing of the NS3 protein also occurred in another isolate of HCV 1b strain. However, a processing of NS3 derived from isolate K seems to be a little less efficient than that from isolate JS (Fig. 3A, lanes 3 and 6), presumably resulting from the difference in amino acid sequence between them.

Shoji *et al.* (1999) could not detect NS3a-1 and NS3a-2 proteins in 293T cells and insect cells, which may stem from the following reasons: (1) since the internal cleavage of NS3 protein was analyzed without NS4A in 293T cells, NS3a-1 and NS3a-2 might not be generated from NS3 protein; and (2) when full-length HCV cDNA derived from HCV 1b strain (NIH J1) was expressed in insect cells, amino acid of P1 position in the internal cleavage site (DVSVIPT<sup>1428</sup>/S<sup>1429</sup>) is not threonine but alanine in NIH

J1 isolate (Fig. 3B). From our immunoprecipitation data using site-directed mutant, pNS34A-M13 (Thr → Ala at P1 position) (Fig. 3A), it is reasonable to assume that NS3a-1 will not be generated in NIH J1 isolate. Although the other internal cleavage site (HLIFCH<sup>1395</sup>/S<sup>1396</sup>) is conserved in NIH J1 isolate, it is likely that the internal cleavage of NS3 protein by NS34A protease may occur inefficiently in insect cells.

### Oncogenic potential of NS3a-1

Although it was previously reported that the artificially truncated N-terminal portion (aa 1027–1281) of the HCV NS3 protein transforms NIH3T3 cells (Sakamuro *et al.*, 1995), the oncogenic potential of full-length NS3 and the naturally truncated form of NS3 has not been addressed. To express the NS3a-1 coding region, NS3a-1 was amplified by PCR with pNS3 plasmid, and NS3a-1-expression plasmid was designated as pNS3a-1. The size of protein expressed from pNS3a-1 in Rat-1 cells corresponded to the size of NS3a-1 derived from autoproteolysis of the NS3 protein and the expression level of NS3a-1 protein was similar to that of NS3 protein (data not shown). As shown in Fig. 4, *in vitro* transformation

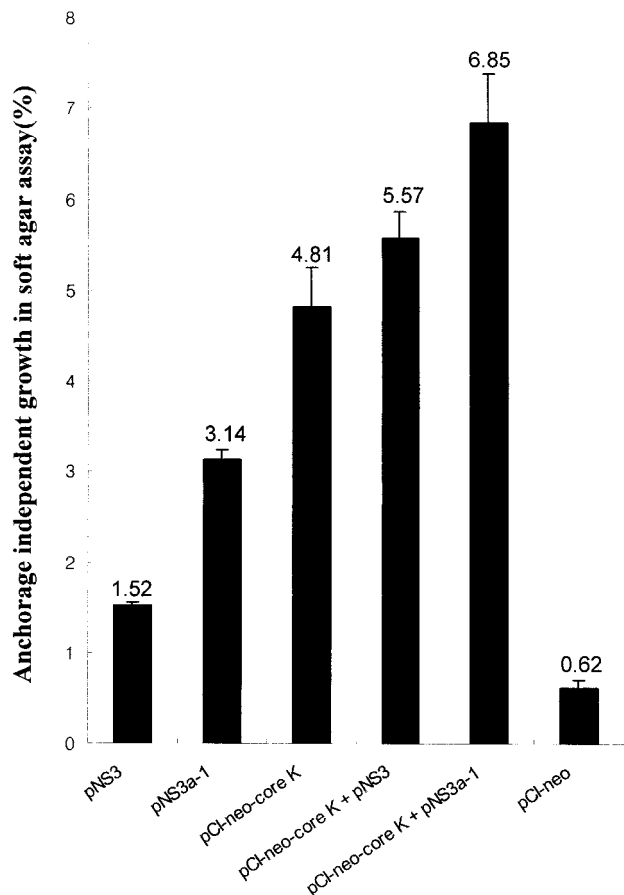


FIG. 4. The oncogenic potential of HCV NS3a-1. Each plasmid was transfected into Rat-1 cells, and a total of  $5 \times 10^3$  Rat-1 cells selected by G418 treatment were used in a soft agar assay. The data are expressed as the percentage of colonies containing more than 200 cells, 2 weeks after plating. Colony numbers represent the means of the three independent experiments and are indicated as numerical values. Error bars = SD.

assay revealed that both NS3 and NS3a-1 have an oncogenic potential and, interestingly, NS3a-1 protein has a twofold higher oncogenic activity than NS3 protein. Since the relative expression level between pNS3 and pNS3a-1 is similar, this difference may be the result of their intrinsic oncogenic potential.

We have previously reported that the HCV core has oncogenic potential which was proportional to the expression level of HCV core protein in Rat-1 cells (Chang *et al.*, 1998). Hence, we investigated the cooperative transformation between NS3 and core protein. Compared to core alone, the coexpression of core and NS3a-1, but not of NS3, led to a statistically significant increase of colony formation ( $P < 0.005$  by Student's *t* test) (Fig. 4), demonstrating that NS3a-1 could increase the oncogenic potential of core protein.

Although it was reported that NS3 and NS3 $\Delta$ C(1027–1459) were localized in both nucleus and cytoplasm, and NS4A is reported to inhibit nuclear localization of NS3 (Muramatsu, *et al.*, 1997), Ishido and Hotta (1998) dem-

onstrated that p53, tumor-suppressor protein, formed a complex with the N-terminus of NS3 (amino acids 1055 and 1200 residues) of HCV, both in the absence and the presence of NS4A. On the basis of these observations, it is possible to speculate that either NS3 or NS3a-1 protein may promote cell transformation through the inhibition of p53 function in cytoplasm.

In addition to the effect of NS3a-1 on the oncogenic potential, it is possible that the internal cleavage of NS3 plays roles in virus replication. Our observation that the internal cleavage sites reside in RNA helicase domain suggests that autoproteolysis of NS3 could affect virus replication by downregulating the quantity of intact RNA helicase. Morgenstern and Thomson (1997) observed a fivefold stimulation of protease activity when the C-terminal helicase domain of NS3 was bound to poly(U). So, HCV serine protease activity could be affected by the internal cleavage at the carboxyl-terminus of NS3. Considering the importance of polyprotein processing of NS3, it would be of interest to compare the catalytic activity of the internally cleaved NS3 products and that of the intact NS3 protein.

## MATERIALS AND METHODS

### Construction of plasmids

To construct expression vectors encoding NS3 (aa 1019–1657), NS34A (aa 1019–1711), and NS3-NS4-NS5 (aa 1019–3010), a cDNA fragment from HCV-1b (JS strain) was used as a polymerase chain reaction (PCR) template (Cho *et al.*, 1998). NS3 sequences were amplified with 5'-GTAAAGCTTCATCTGACTAATACTACA-3' (sense primer) and 5'-CACGTCGACCTAAGTGACGACCTCCAGGTC-3' (antisense primer), and NS34A or NS345 sequences were amplified with sense primer used for amplification of NS3 and antisense primers, 5'-GAGGTCGACCTAGCACTCTTCCATTTCATC-3', or 5'-GCTCTAGATGCGGTTGGGGAGCAGGTA-3', respectively. The *Hind*III and *Sal*I, or *Hind*III and *Xba*I fragments of amplified DNA fragments were subcloned into the pSK vector (Stratagene), and designated as pSK-NS3, pSK-NS34A, and pSK-NS345, respectively. Using these intermediate plasmids, NS3-, NS34A-, and NS345-encoding DNA fragments were introduced into the mammalian expression vector pCI-neo (Promega) and designated as pNS3, pNS34A, and pNS345, respectively. To construct pNS3a-1, NS3a-1 nucleotide sequences were amplified from pNS3 using PCR with sense primer, 5'-GTAAAGCTTCATCTGACTAATACTACA-3' and antisense primer, 5'-GTTCTAGATGGTGGGTATGACGGACACATC-3'. An amplified DNA fragment was cloned into pCI-neo vector using restriction enzymes, *Hind*III and *Xba*I. To construct pNS4A, NS4A was amplified by PCR with following oligonucleotides as primers: sense primer, 5'-GGCCATGGGAAGCAGTTGGGTGCTAGTAGGC-3' and antisense primer, 5'-GCTCTAGACGCACTCTTCCATTTCATCGAAC-

3'. The *NcoI* and *XbaI* fragment of the amplified DNA fragment was subcloned to pCI-neo vector, and the resulting plasmid was designated as pNS4A. To construct pNS34A-K (aa 1019–1711), a cDNA fragment of HCV-1b (K isolate) (Cho *et al.*, 1993) was used as a PCR template. NS34A nucleotide sequences of HCV-1b (K isolate) were amplified with 5'-CCGCCATGGAAGGGCAGGGTGGCG-3' (sense primer) and antisense primer used for amplification of NS4A, and the amplified DNA fragment was subcloned to pCI-neo using restriction enzymes, *NcoI* and *XbaI*.

To inactivate NS3 serine protease and generate *SacII* site, Ser<sup>1164</sup> and Ser<sup>1165</sup> in NS3 region were mutated to Ala and Ala in pSK-NS34A using specific primers (sense primer, 5'-TGAAGGGCGCCGCGGGTGGTC-3'; antisense primer, 5'-GACCACCCGCGCGCCCTTCA-3') and a PCR-based site-directed mutagenesis kit (Stratagene). The resulting PCR fragment was subcloned to pCI-neo expression vector, and designated as pNS34A-M1. Site-directed mutant, pNS34A-M1 was confirmed by digestion of generated *SacII* site (underlined). The other 14 site-directed mutants (from pNS34A-M2 to pNS34A-M15) with two amino acid mutations were constructed according to procedures previously described and confirmed by digestion of generated *SphI* or *XbaI* site (underlined in used primers). The oligonucleotide sequences of the primers used for PCR-based site-directed mutagenesis were as follows: pNS34A-M2, 5'-GCCGATGGTG-GTGCATGCGGGGGCGCCTAC-3' (sense) and 5'-GTAGGCGCCCCCGCATGCAACCACCATCGGC-3' (antisense); pNS34A-M3, 5'-CTCAACTGACTCAGCATGCATCTTGGG-CATTG-3' (sense) and 5'-CAATGCCACGCTGCATGCT-GAGTCAGTTGAG-3' (antisense); pNS34A-M4, 5'-GGA-AGACATCTCCCCGGGTGCCATTCCAAG-3' (sense) and 5'-CTTGGAATGGCACCCGGGGAGATGTCTTCC-3' (antisense); pNS34A-M5, 5'-CTCATTTTCTGCCCGGGA-AGAAGAAGTCT-3' (sense) and 5'-AGACTTCTTCTT-CCCCGGGCGAGAAAATGAG-3' (antisense); pNS34A-M6, 5'-TGCCATTCCAAGCCCGGGTCTGACGAGCTC-3' (sense) and 5'-GAGCTCGTCAGACCCGGGCTTGGAATGGCA-3' (antisense); pNS34A-M7, 5'-GGCGAGCTCGTCGCATGC-CTTCTTGGAATG-3' (sense) and 5'-CATTCCAAGAAG-GCATGCGACGAGCTCGCC-3' (antisense); pNS34A-M8, 5'-AAGAAGAAGTCTCCCCGGGCTCGCCGCAAAG-3' (sense) and 5'-CTTTGCGGCGAGCCCGGGAGACTTCT-TCTT-3' (antisense); pNS34A-M9, 5'-TCTGACGAGCTC-CCCCGGGAAGCTGTCAGCC-3' (sense) and 5'-GGCTGA-CAGCTTCCCCGGGAGCTCGTCAGA-3' (antisense); pNS34A-M10, 5'-CTCGCCGCAAAGCCCGGGGCCCTCGGACTT-3' (sense) and 5'-AAGTCCGAGGGCCCCGGGCTTTGCGG-CGAG-3' (antisense); pNS34A-M11, 5'-CTCGCCGCA-AAGGCATGCGCCCTCGGACTT-3' (sense) and 5'-AAG-TCCGAGGGCGCATGCCTTTGCGGCGAG-3' (antisense); pNS34A-M12, 5'-AAGCTGTCAGCCCCGGGCTTAATGC-TGTA-3' (sense) and 5'-TACAGCATTAAGCCCGGGGCT-GACAGCTT-3' (antisense); pNS34A-M13, 5'-GTCCGT-

CATACCGGCATGCGGAGACGTCGTTG-3' (sense) and 5'-CAACGACGTCTCCGCATGCCGGTATGACGGAC-3' (antisense); pNS34A-M14, 5'-CTATGACGCGGGCGCATGCT-GGTATGAGCTC-3' (sense) and 5'-GAGCTCATACCA-GCATGCGCCCCGCGTCATAG-3' (antisense); pNS34A-M15, 5'-CACGCCTGCTGAGGCATGCGTTAGGTTGCGG-3' (sense) and 5'-CCGCAACCTAACGCATGCTCAGCAGGCGTG-3' (antisense).

## Cells and DNA transfection

COS-7 cells and NIH3T3 cells, which were cultured in DMEM with 10% fetal bovine serum, were transfected with expression plasmids by CaPO<sub>4</sub> coprecipitation method as described previously (Lowy *et al.*, 1978). Human hepatoblastoma HepG2 cells grown in DMEM with 10% fetal bovine serum were transfected with plasmid DNAs using a Lipofectin-mediated method according to the manufacturer's protocols (GIBCO BRL). Rat-1 cells (5 × 10<sup>5</sup> cells per 60-mm-diameter dish) were transfected with 10 μg of pCI-neo, NS3-, or core-encoding plasmids and cotransfected with combination of NS3-encoding plasmids and pCI-neo-core K by CaPO<sub>4</sub> coprecipitation method.

## Radiolabeling and immunoprecipitation

COS-7, NIH3T3, or HepG2 cells transfected with expression plasmids were radiolabeled with 100 μCi of [<sup>35</sup>S]methionine (Dupont, NEN) for 4 h after a 1-h incubation in methionine and cysteine-free media. Labeled cells were rinsed with cold phosphate-buffered saline and incubated with cold lysis buffer (50mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.02% sodium azide; 0.1% SDS; 100 μg/ml PMSF; 1 μg/ml aprotinin; 1% Nonidet-P40; 0.5% sodium deoxycholate) on ice for 20 min. Cell lysates were centrifuged at 12,000 *g* for 10 min at 4°C and clarified lysates were incubated with pooled HCV-infected human sera or antiprotease antibodies against recombinant protein of the N-terminal portion of NS3 (aa 1027–1218) expressed *E. coli* on a rocker at 4°C overnight. Pansorbin cells (Chemicon) containing protein A were added and incubated under the same conditions for 2 h. Beads were washed thoroughly with washing buffer and resuspended in SDS-PAGE loading buffer. Samples were heated at 100°C and resolved on SDS-12% or SDS-12.5% polyacrylamide gels for analysis by autoradiography.

## Soft agar assay

Cell colonies harvested after G418 selection (400 μg/ml) for 10 days were tested for anchorage-independent growth in soft agar by a procedures described earlier (Chang *et al.*, 1998). Briefly, G418-resistant colonies (more than 3000) were pooled, and media containing 0.25% agarose (Sigma) were inoculated with 5 × 10<sup>3</sup>

cells. At 14 days of cultures, the number of colonies (more than 200 cells) were counted.

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